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Source: *The Journal of Cell Biology*, Vol. 106, No. 3 (Mar., 1988), pp. 773-778
Published by: The Rockefeller University Press
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Nuclear Migration in a *nud* mutant of *Aspergillus nidulans* Is Inhibited in the Presence of a Quantitatively Normal Population of Cytoplasmic Microtubules

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Abstract. Nuclear migration was studied in germinating conidia of a temperature-sensitive mutant of the fungus *Aspergillus nidulans*. At the restrictive temperature motility was demonstrably impaired because significantly fewer nuclei migrated into the germ tube relative to a population of similarly sized germlings grown at the permissive temperature. Further comparison of these populations showed that the mutant was leaky in that an increasing number of nuclei migrated as the total nuclear content increased in each germling. The restrictive temperature also induced elevated mitotic asynchrony and increased numbers of nuclei per germling. Serial section–based reconstruction of the microtubules in a freeze-substituted germling showed that they were not attached to the nucleus-associated organelles, were approximately parallel to the long axis of the germ tube, and seemed to be randomly distributed between the central and peripheral cytoplasm. Five germlings from each temperature were selected for quantitative analysis of cytoplasmic microtubules. All 10 germlings had typical nuclear migration phenotypes. No significant temperature-related difference in microtubule density was found. We conclude that inhibition of nuclear migration in the mutant is the effect of some defect other than the failure of cytoplasmic microtubules to assemble to their normal population density. We also suggest that nuclear motility is not dependent on mitosis-related microtubules.

ORGANELLE motility is essential for the normal functioning and development of most eukaryotic cells. Many studies show that microtubules are important components of diverse motile systems (reviewed by Schliwa, 1984). This is true for nuclear migration in fungi where morphology, inhibitor, and mutational studies combine to show that undamaged and properly oriented microtubules are needed for normal motility (reviewed by McKerracher and Heath, 1987). However, the way in which the microtubules are involved is unclear. Germinating conidia of the fungus *Aspergillus nidulans* (Eidam) Winter are good cells in which to study the role of microtubules in nuclear migration. The uninucleate conidium germinates to produce a germ tube, and in these germlings mitosis and nuclear migration combine to establish and maintain an essentially uniform distribution of nuclei. Oakley and Morris (1980) showed that nuclear motility is dependent on β-tubulin, probably in the form of microtubules, and is independent of mitosis. They (Oakley and Morris, 1980; Oakley and Rinehart, 1985) also showed that nuclear motility depended on the products of nuclear distribution (*nud*) genes, which are nonallelic with α and β tubulin genes. The natures of the temperature-sensitive mutant *nud* gene products are unknown, but they could function by altering the numbers, lengths, distribution, or stabilities of the motility-related microtubules. Such phenotypic changes can only be reliably determined by quantitative electron microscopy of freeze-substituted cells. Immunofluorescence localization of microtubules in the small, cell wall–bound germlings is unreliable because it involves fixations and extractions of questionable reliability and lacks adequate resolution, and electron microscopy using conventional fixations is reportedly inadequate for microtubule analysis in some fungi (Howard and Aist, 1979; Heath and Rethoret, 1982; Hoch and Staples, 1983; McKerracher and Heath, 1985). The nature of the *nud* gene products, and therefore further characterization of the microtubule-based motility system, may be determined by analysis of the microtubules in the inhibited germlings. This paper reports a quantitative study comparing the phenotype of a *nud* mutation at nuclear-motility permissive and nuclear-motility restrictive temperatures.

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Materials and Methods

Culture Preparation

A stock culture strain, ts 320, of *Aspergillus nidulans* was generously provided by Dr. N. R. Morris (Robert Wood Johnson Medical School, Piscataway, NJ) and maintained on YAG medium (0.5% yeast extract, 1.9% agar, 2% glucose). This strain was isolated by Morris (1976) and carries mu-

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tation *nud*B2 in a Fungal Genetics Stock Center 154 background. For light and electron microscopic examination, conidia from 6-d-old cultures were suspended in sterile distilled water and diluted to ~70,000 conidia/ml. Pieces of dialysis membrane were autoclaved in liquid YAG, dipped in boiled 0.5% aqueous locust bean gum, placed onto YAG in petri dishes, and inoculated with conidial suspension. Germlings were grown at the permissive temperature for 14-15 h at room temperature (~25°C). To grow germlings at the restrictive temperature, the cultures were placed in an incubator that was initially at room temperature but timed to heat to 45°C between 3 and 4 h after inoculation. These germlings were fixed after 7-8.5 h growth at 45°C.

Light Microscopy

To reliably measure the number and position of nuclei, germlings grown on dialysis membrane were fixed in 5% glutaraldehyde in 0.067 M phosphate buffer, rinsed two to three times in buffer, stained for ~15 min in 200 μg/ml mithramycin in 300 mM $MgCl_2$, and mounted either in the stain or in Citifluor (Marivac Ltd., Halifax, Nova Scotia). The fluorescent nuclei in the germlings were photographed with a Reichert Polyvar microscope equipped with epifluorescence illumination using a B1 filter cube and a 100× 1.32 NA objective lens. The presented data are the pooled results from three separate experiments at each temperature.

Electron Microscopy

Germlings were prepared for freeze substitution as described previously (Howard and Aist, 1979; Heath and Rethoret, 1982). Briefly, the germlings on dialysis membrane were frozen in liquid propane, substituted in 2.5% osmium tetroxide in acetone for 2 d at −80°C, and then warmed for 1 h at −20°C, 1 h at 4°C, and 1 h at room temperature. This was followed by four changes of acetone (dried over $CuSO_4$). The specimens were then either embedded in Epon after an acetone/Epon mixture, or embedded in Spurr's resin (Spurr, 1969) after infiltration via ethanol and propylene oxide. Some germlings were embedded while still attached to the dialysis membrane, while others were removed. Embedment was between glass slides coated with Liquid Release Coating (Reymond and Pickett-Heaps, 1983).

Nomarski differential interference contrast optics were used to preselect germlings of the appropriate size and with no obvious ice crystal damage. The germlings were serially sectioned longitudinally and the numbers and positions of nuclei determined with the electron microscope.

Analysis of Microtubules

To determine the general organization of microtubules in the germlings, serial sections of one of the germlings grown at permissive temperature were photographed and the micrographs were printed at a final magnification of 59,500. A montage of each section was prepared and the microtubules were traced onto an acetate sheet. The tracings were digitized and a stereopair diagram of the microtubule population was produced as described previously (Moens and Moens, 1981).

To measure the effect of the mutation on the abundance of microtubules, a modification of the procedure of Heath and Heath (1978) was used. Montages (at a final magnification of 59,500) were prepared of the entirety of each of three sections from each germling: one from an approximately median section and two from sections located 9-10 sections on either side of median. An acetate grid with parallel lines 2.5-cm apart was laid over each montage with the lines oriented at right angles to the long axis of the germ tube. When a germ tube was highly curved, the acetate sheet was reoriented so that the lines were perpendicular to the long axis of the portion of the germ tube being analyzed. The number of microtubule profiles intersected by the grid lines were counted in each germ tube, and the length of the test line over cytoplasm in each germ tube was measured with a digitizer. All organelles were excluded from the cytoplasm measurements. The total number of microtubule-grid line intersections divided by the total length of measured cytoplasm from all three sections of a germling were added together to give an estimate of the microtubule density per germling.

Results

Nuclear Behavior

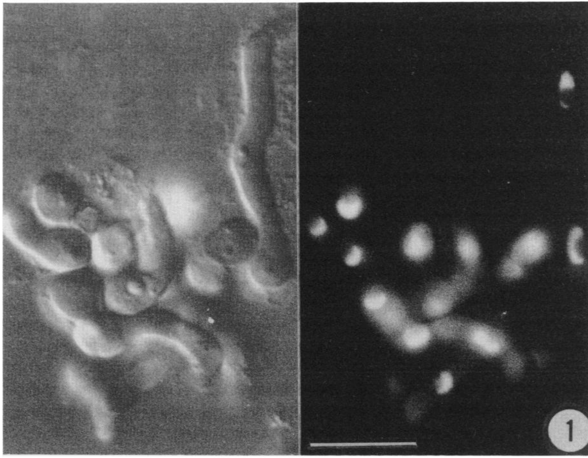
Preliminary observations showed that germling lengths (germ tube plus conidium) of between 15 and 45 μm were optimal for our analyses because shorter germlings typically contained only a single nucleus that had not migrated from the conidium, even at the permissive temperature, whereas longer ones were difficult to use for high magnification electron microscopy of entire germlings. Because germlings grew faster at the higher restrictive temperature, shorter incubation times were selected relative to the permissive temperature in order to produce populations with similar germ tube lengths (Table I) and, therefore, at comparable stages of development. The characteristics of the two populations studied are shown in Table I with representative germlings illustrated in Figs. 1 and 2. As shown in Fig. 3, the migratory behavior of the nuclei in these two populations differed, with 95% of germlings showing some degree of nuclear migration at the permissive temperature versus only 58% at the restrictive temperature. At the restrictive temperature, even when some nuclei had migrated, there were typically fewer that had moved out of the conidium, so that there were proportionally many more (79 vs. 12%) germlings with over half of their nuclei still in the conidium (Fig. 3). In both populations of germlings, the number of nuclei migrating into the germ tubes was significantly ($p < 0.001$) linearly correlated with the number of nuclei present (Fig. 3) but the slope of the linear regression line was significantly ($p < 0.001$ using analysis of covariance) lower (i.e., fewer nuclei migrated into the germ tube) at the restrictive temperature. In addition to differing in nuclear migration behavior, the restrictive temperature population contained significantly ($p < 0.001$, Student's t test) more nuclei (Table I) in germlings whose average size was comparable to the permissive temperature population. The restrictive temperature also induced a substantial increase in the number of germlings containing nuclear numbers indicative of asynchronous mitoses (Table I).

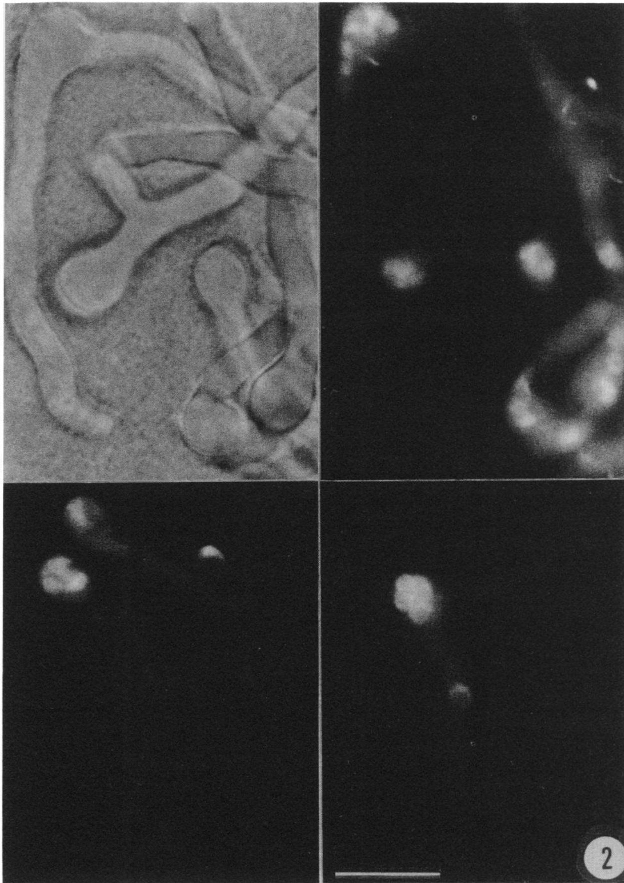
Table I. Characteristics of Germlings at Permissive and Restrictive Temperatures

Characteristics	Permissive	Restrictive
Sample size	86	163
Mean germ tube length (*μm*)	28.5 ± 8.0	26.7 ± 7.4
Germling length range (*μm*)	15.5–44.7	15.5–43.0
Mean no. of nuclei per germling	2.94 ± 1.00	3.49 ± 1.32
Range of no. of nuclei per germling	1–5	1–8
Germlings with synchronous mitosis (i.e., containing 1, 2, 4, or 8 nuclei)	91%	57%
Germlings with asynchrony after the second mitosis (i.e., containing 3 nuclei)	8%	31%

Microtubule Behavior

Before quantitative analysis of the cytoplasmic microtubules, we had to determine the orientation and distribution pattern of microtubules in order to select an effective and valid analytical procedure. To do this we selected a single permissive temperature-grown germling showing a normal phenotype (cell *A* in Table II and Fig. 3) and reconstructed its entire population of cytoplasmic microtubules. All seven microtubules were aligned approximately parallel to the long axis of the germ tube and were not preferentially accumulated in either the peripheral or cortical cytoplasm (Fig. 4). None were





Figures 1 and 2. (Fig. 1) Cluster of germlings grown at the permissive temperature. Comparison of the Nomarski differential interference contrast image with the distribution of nuclei shown by mithramycin fluorescence shows that several germlings are binucleate and that at least one nucleus of each has migrated into the germ tube. Bar, 10 μm. (Fig. 2) Germlings grown at the restrictive temperature. In both the clustered group with comparative Nomarski and mithramycin fluorescence images and the three other mithramycin-stained cells, the bulk of the nuclei are clearly restricted to the conidia. Bar, 10 μm.

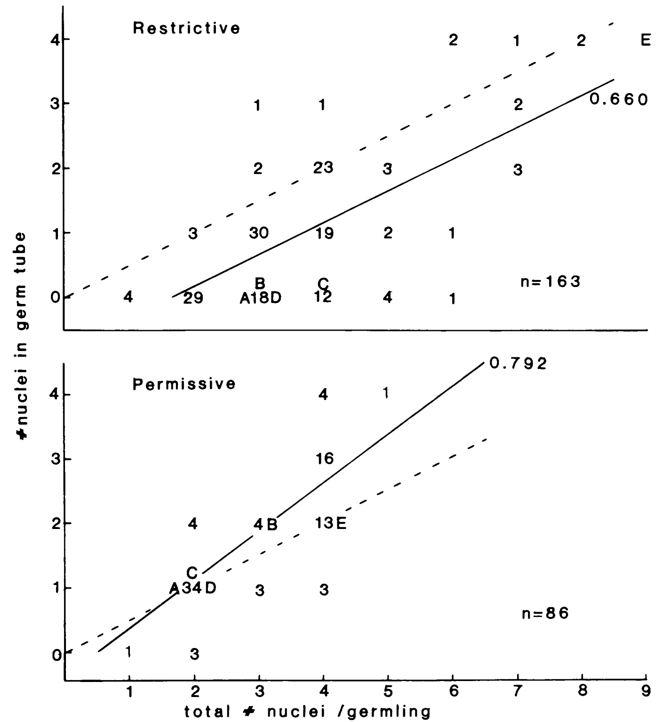


Figure 3. Comparison of permissive and restrictive temperature–grown populations of germlings whose other characteristics are given in Table I. In each population, the number of germlings showing a particular combination of total and migrated nuclei are indicated. The linear regression lines with correlation coefficients are shown for each population. The broken lines show the level at which half of the total number of nuclei per germling had migrated into the germ tube and are included to facilitate comparison between the two populations. The letters show the nuclear condition of the cells analyzed in Table II.

Table II. Density of Cytoplasmic Microtubules at Permissive and Restrictive Temperatures

Cell designation	Nuclear status (total nuclei/germling per nuclei migrated into germ tube)*	Germling length	Microtubule intersections per μm transected cytoplasm
		μm	
Permissive			
A	2/1	25.9	0.0575
B	3+/2	25.4	0.0885
C	2/1	17.9	0.0000
D	2/1	21.7	0.0168
E	4/2	22.5	0.0806
Mean ± SD		22.7 ± 3.2	0.049 ± 0.039
Restrictive			
A	3+/0	17.0	0.0565
B	3+/0	15.8	0.0304
C	4+/0	30.8	0.1380
D	3+/0	25.7	0.0909
E	9/4	29.9	0.0559
Mean ± SD		23.8 ± 7.1	0.074 ± 0.042

* Nuclear values with a plus sign indicate the difficulty in counting nuclei that are densely packed in the conidia. In each case small discontinuities in the series of sections precluded absolute counts. There were at least the indicated number and possibly one more in each cell.

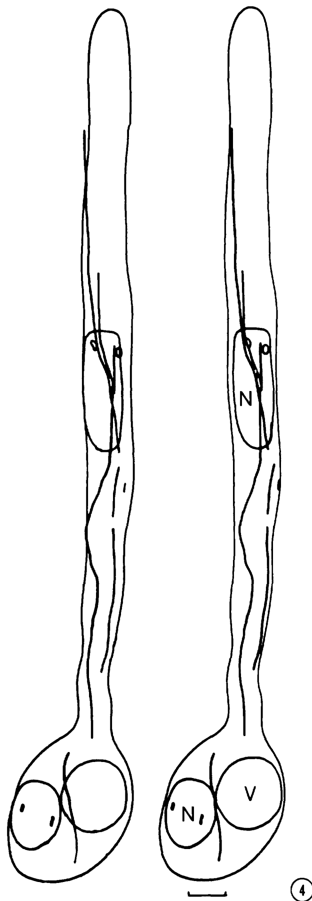


Figure 4. Stereopair of germling *A* from the permissive temperature population listed in Table II. The microtubules are shown by single lines inside the cell and the outlines of the two nuclei (*N*), vacuole (*V*) and the germling wall are indicated by single lines taken from near median sections. The two small bars on the lower nucleus and the small circles on the upper nucleus represent the NAOs of these mitotic nuclei. The spindles ran between the NAOs. Since the cell outline is represented by a single line derived from an approximately median section, the tilted image on the left produces the effect of narrowing the cell outline such that the end of one microtubule lying close to the plasmalemma artefactually appears outside of the cell. Bar, 1 μm.

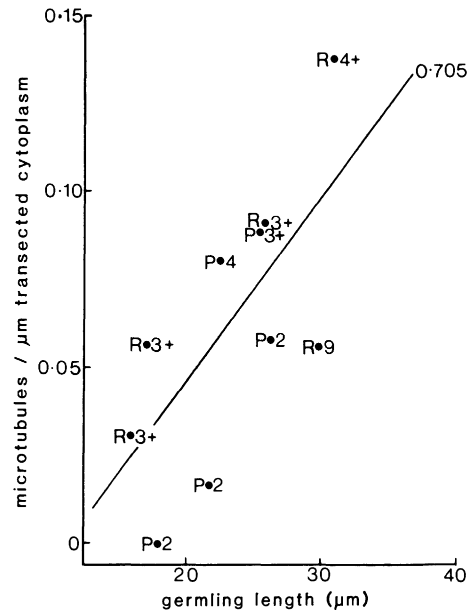


Figure 5. Illustration of the correlation between germling length and microtubule density found in the cells listed in Table II. For each point, the incubation temperature (permissive or restrictive) and the total number of nuclei are given to facilitate cross-reference to Table II. The linear regression line and correlation coefficient are indicated.

intimately associated with the nucleus-associated organelles (NAOs),[1] although several were close to the nuclear envelope of the migrated nucleus, and only one end of one microtubule lay close to the plasmalemma. However, the microtubules were predominantly concentrated away from the tip of the germ tube. Based on these observations, sampling of the entire germ tube cytoplasm from consistently selected, near-median longitudinal sections should give a reasonable estimate of the microtubule population in the region of nuclear migration. We sampled five germlings from each temperature population. Based on the data in Fig. 3, we were able to identify germlings that closely fitted the population norm (permissive temperature population) or showed the most extremely inhibited phenotype (cells *A–D* in the restrictive temperature population). We selected the fifth cell (*E*) from the restrictive temperature because it contained more nuclei but still lay close to the linear regression line for the population (Fig. 3). The mean germling lengths of the two sampled populations were very similar, but, as in the total populations, the restrictive temperature cells contained more nuclei (Table II). The density of microtubules showed no significant difference (Student's *t* test) between the two populations (Table II). However, there was a significant ($p = 0.01$) positive correlation between germling length and microtubule density (Fig. 5). Interestingly, germling *A* from the permissive temperature was the only one containing dividing nuclei (metaphase) and its microtubule density was very close to the linear regression line; it did not contain an elevated number of microtubules associated with mitosis.

Discussion

Our results confirm previous data (Morris, 1976; Oakley and Morris, 1980; Oakley and Rinehart, 1985) showing that the *nud*B2 mutation disrupts normal nuclear migration at the restrictive temperature. We have extended the description of the phenotype by showing that the degree of "leakiness" (i.e., degree of migration) increases with nuclear number and that the coordination between germ tube length and number of nuclei is altered at the restrictive temperature. We have also shown that there is a degradation of mitotic synchrony in the mutant at the restrictive temperature. This result is contrary to the data of Morris (1976), who noted synchronous mitosis in hyphae incubated for 4 h at the restrictive temperature, and may indicate some unsuspected difference between hyphae and germlings. The significance of these additional phenotypic features will become more apparent in future studies and will be returned to briefly in the following discussion.

Nuclear motility in *Aspergillus nidulans* is known to be dependent on microtubules (Oakley and Morris, 1980; Oakley and Rinehart, 1985), most likely cytoplasmic microtubules. Our observation that longer germ tubes contain more cytoplasmic microtubules supports the idea that these microtubules are indeed involved in nuclear migration, because longer germ tubes are likely to require more motility-generating elements to move more nuclei through more cytoplasm. The simplest hypothesis, based on previous data, for the mode of action of the *nud*B2 gene product is that it causes loss of microtubules at the restrictive temperature. Such is

1. *Abbreviation used in this paper*: NAO(s), nucleus-associated organelle(s).

clearly not the case, because cells showing the restrictive-temperature phenotype contain a quantitatively normal population of interphase cytoplasmic microtubules, which are the population most likely to be involved in nuclear migration. Consequently, we conclude that the *nud*B2 gene product is not involved in regulation of the quantity of polymerized tubulin in the cell. We cannot rule out the possibility that the microtubules that are found at the restrictive temperature are hyperstable as reported for the mitotic microtubules produced by the *ben*A33 gene, which also blocks nuclear migration (Oakley and Morris, 1981). However, agents such as taxol and D_2O that cause hyperstabilization of microtubules are also known to increase microtubule polymerization (Dustin, 1984). The absence of such an increase in our results is an argument, albeit a weak one, against the hyperstabilization hypothesis. We believe that it is more likely that the *nud*B2 gene product is involved as an intermediate between the apparently normal population of cytoplasmic microtubules and either the nuclei or other components of the cytoskeleton, but this cannot be substantiated by thin section techniques in this material because such linkages have not been described with convincing regularity.

It is possible that the *nud*B2 gene causes changes in the organization of microtubules at the restrictive temperature. Because we have shown that the permissive temperature microtubule population shows no clear regular organizational pattern it is very difficult to devise a valid and sensitive quantitative assay for subtle changes in organization. Such an assay would be the only way of ruling out potentially important small changes in distribution. However, there are reports of gross changes, such as close packing of microtubules induced by nocodazole and fluorodeoxyuridine (Heath, 1982), griseofulvin (Gull and Trinci, 1974), and elevated temperature (Rieder and Bajer, 1978), which would have been detected in our material and did not occur. Likewise gross translocations exclusively to the central or peripheral cytoplasm would have been detectable but were not observed. Within these limitations, we conclude that major rearrangement of microtubules does not occur at the restrictive temperature. There is evidence in the literature to suggest that fungal nuclear motility may be generated by lateral interactions between microtubules and the nuclear envelope (e.g., Heath and Heath, 1978). Such is also quite likely in *Aspergillus nidulans*. For example, the microtubules passing over the nucleus in the germ tube in Fig. 4 were closely associated with the nuclear envelope. The detailed analysis of complete serial sections of numerous germlings necessary to detect possible temperature-induced changes in such an association is beyond the scope of this work. If the *nud*B2 gene does cause a change in microtubule–nuclear envelope associations, it would strengthen our suggestion that the gene product is an intermediate between the microtubules and the nuclei. However, the observed pattern of microtubules in the permissive temperature is also consistent with previous suggestions (McKerracher and Heath, 1986 and 1987) that cytoplasmic microtubules play an indirect role in nuclear motility rather than interacting directly with nuclear envelopes or the NAOs.

The most serious limitation to the interpretation of our data is that nuclear migration may be a transient event involving a transient population of microtubules, an event that we have failed to observe in our limited random samples of germlings. There is certainly abundant evidence for mitosis-specific nuclear motility mediated by NAO-associated astral (i.e., cytoplasmic) microtubules in other fungi (reviewed in Heath, 1978 and 1981, and Aist and Berns, 1981) and our data do show that migration into the germ tube normally occurs after the first mitosis (i.e., in the binucleate germlings). Because it is most likely that the hypothetically active astral microtubules are either only formed (e.g., Heath and Heath, 1976) or only functional (e.g., Aist and Berns, 1981) during anaphase or telophase, stages which occupy $\sim$1% (certainly $<$5%) of the nuclear cycle (Bergen and Morris, 1983), it would be very difficult to accumulate enough well frozen cells of these stages for ultrastructural analysis after freeze substitution. It is equally likely to be as difficult to locate a gene product that may be functional for such a short portion of the cell cycle. Furthermore, direct light microscopy of living cells in order to detect transient nuclear movements is impractical because the nuclei in the germlings are not normally detectable with either phase or Nomarski interference contrast optics. However, we do know that mitotic nuclei of *Aspergillus* lack (Fig. 4) or have very few astral microtubules (Gambino et al., 1984). This observation, combined with the demonstration that the *nud* mutants can complete mitosis in the absence of nuclear motility (Morris, 1976; Oakley and Morris, 1980), and the observation that in *Fusarium* the abundant astral microtubules apparently play a major role in mitotic force generation (Aist and Berns, 1981) suggest that astral microtubules are unimportant in *Aspergillus nidulans* and that nuclear motility is not dependent on mitosis-related microtubules.

In some respects our observations of the microtubules in the mitotic germling shown in Fig. 4 differ from previous reports of microtubules in this species. For example, Gambino et al. (1984) showed that most cytoplasmic microtubules were either depolymerized or became fixation labile during mitosis in protoplasts. While it is possible that there are behavioral differences between protoplasts and germlings, we feel that it is more likely that the fixation and staining protocols used by Gambino et al. (1984) failed to preserve or detect cytoplasmic microtubules that changed their characteristics at mitosis. It is most likely that our techniques are indeed superior for presevation of cytoplasmic microtubules and that the complement shown in Fig. 6 are a true indication of the in vivo situation. The fact that the population density of cytoplasmic microtubules in the metaphase cell (permissive, cell *A* in Table II) was very close to the mean of all cells, all others of which were in interphase, supports the hypothesis that interphase cytoplasmic microtubules do indeed persist tnroughout the nuclear cycle and therefore suggests that their absence in the work of Gambino et al. (1984) is indeed an indication of a change to greater fixation lability. However, the absence of NAO-associated microtubules is surprising, since such have been detected, albeit in very low numbers, on both mitotic (Gambino et al., 1984; Oakley and Morris, 1983) and interphase (Oakley and Morris, 1980) NAOs of this species. As discussed above, it is most likely that there are nuclear cycle–dependent changes in NAO-associated microtubules; consequently, their absence in Fig. 4 is most likely a stage-specific phenomenon associated with the early metaphase stage shown by that cell. A detailed analysis of this phenomenon has not been attempted and is irrelevant to the basic conclusion that the *nud*B2 gene does not exert its

inhibitory effect on nuclear motility by reducing the population density of cytoplasmic microtubules.

We thank Dr. P. B. Moens and Barbara Spyropoulos for assistance with computerized analysis of microtubule arrangement and density, and Drs. B. R. Oakley. K. L. O'Donnell, R. J. Howard, and N. R. Morris for discussions that have contributed to this work.

We acknowledge the Natural Sciences and Engineering Research Council of Canada for financial support.

Received for publication 1 October 1987, and in revised form 27 November 1987.

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